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Effects of Ozone on Leukocyte DNA

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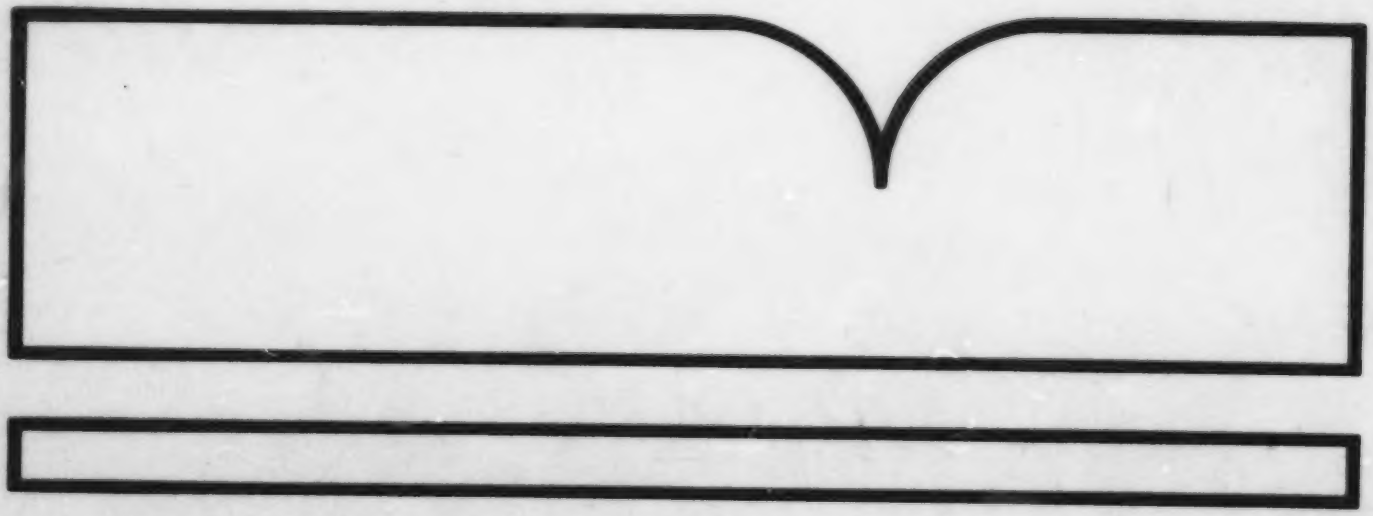

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EFFECTS OF OZONE ON LEUKOCYTE DNA

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Health Effects Research Laboratory
Office of Research and Development
U. S. Environmental Protection Agency
Research Triangle Park, North Carolina 27711

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16. ABSTRACT

This research program was initiated with the overall objective of determining whether exposure to ozone could damage the DNA of peripheral blood cells.

An animal model system was designed in which glycogen was used to stimulate the production of peritoneal exudate cells (PECs) in mice. These PECs were labeled by repeated i.v. injection of ^{3}H-thymidine. The labeled PECs circulated briefly through the peripheral blood and eventually accumulated in the pertionela cavity. The experimental animals were either exposed to ozone or ambient atmosphere (sham treated) during the period of time when the PECs were circulating through the perpheral blood. These PECs were then harvested and their DNA analyzed for single strand breaks on alkaline sucrose gradients.

At very high levels of ozone exposure (5 ppm 9800 ug/m^3 24 hours) DNA damage was readily apparent. At lower levels of ozone (1 ppm for 24 hours), some DNA damage may have occurred. In each experiment at this level, some decrease in the average molecular weight of the DNA from ozone exposed animals was detedted. However, the data were not statistically significant in terms of increased number of single strand breaks. No attempt was made to assess DNA damage at even lower ozone levels.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Hea'+h Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of heatlth care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The U. S. Environmental Protection Agency is concerned with the possible effects ozone exposure could have on DNA. This report addresses whether exposure to ozone can cause damage to the DNA of peripheral blood cells.

F. G. Hueter, Ph.D.
Director
Health Effects Research Laboratory

ABSTRACT

This research program was initiated with the overall objective of determining whether exposure to ozone could damage the DNA of peripheral blood cells.

An animal model system was designed in which glycogen was used to stimulate the production of peritoneal exudate cells (PECs) in mice. These PECs were labeled by repeated i.v. injection of ^{3}H-thymidine. The labeled PECs circulated briefly through the peripheral blood and eventually accumulated in the peritoneal cavity. The experimental animals were either exposed to ozone or ambient atmosphere (sham treated) during the period of time when the PECs were circulating through the peripheral blood. These PECs were then harvested and their DNA analyzed for single strand breaks on alkaline sucrose gradients.

At very high levels of ozone exposure (5 ppm [9800 $\mu g/m^3$] for 24 hours) DNA damage was readily apparent. At lower levels of ozone (1 ppm for 24 hours), some DNA damage may have occurred. In each experiment at this level, some decrease in the weight average molecular weight of the DNA from ozone exposed animals was detected. However, the data were not statistically significant in terms of increased number of single strand breaks. No attempt was made to assess DNA damage at even lower ozone levels.

Thus, this project raises the possibility that some damage may occur to the DNA of peripheral blood cells during exposure to environmentally realistic levels of ozone. Unfortunately, the currently available technology for assaying DNA damage is not sufficiently sensitive to unambiguously resolve this question.

This report was submitted in fulfillment of Grant Number R805114 by the University of North Carolina under the sponsorship of the Environmental Protection Agency. This report covers the period October 15, 1977 to October 17, 1979, and work was completed as of October 17, 1979.

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LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

DNA	-- deoxyribonucleic acid
TCA	-- trichloroacetic acid
BSA	-- bovine serum albumin
EDTA	-- ethylenediaminetetroacetic acid
PBS	-- phosphate buffered saline
PECs	-- peritoneal exudate cells

SECTION 1

INTRODUCTION

In addition to its direct effects on lung tissue, ozone has been shown to cause a number of systemic effects such as changes in visual acuity, sphering of red blood cells, and alterations in serum enzyme levels (1-3). Many of these systemic effects are radiomimetic. That is, they appear to mimic the type of damage caused by ionizing radiation, and thus are assumed to be caused by the generation of free radicals within the target cell.

This project was concerned with one such example of systemic damage. Previous investigators have observed that ozone causes chromosome abberations in peripheral blood lymphocytes of both Chinese hamsters (4) and human KB cells (5). This implies, of course, that ozone causes considerable DNA damage in these cells. If this is true, it provides an extremely important avenue of research into the mechanism of ozone toxicology which has not been followed up to date.

Another unresolved question is the manner in which ozone causes systemic damage. Ozone itself is very reactive and might be expected to react primarily with the lung tissue without penetrating any further. It is widely assumed, therefore, that ozone does not cause systemic damage directly, but via production of peroxides and ozonides which migrate to the target cell and generate free radicals. For the most part, this mechanism is speculative, although it has been shown that ozonides and peroxides are produced in response to ozone exposure (6, 7) and that they can cause some of the observed effects in cell culture (8, 9).

One method of assessing ozone related damage to the DNA is to measure the extent of DNA repair synthesis. Cultured lymphocytes have previously been shown to carry out repair of damage to their DNA caused by UV radiation, ionizing radiation, or alkylating agents (10-12). This repair DNA synthesis is quite specific and easy to measure. In addition, the magnitude of the repair synthesis is proportional to the extent of the damage. Lymphocytes, in fact would appear to provide an ideal system for studying DNA repair, since normal replicative DNA synthesis is quite low in unstimulated lymphocytes (10).

Since most examples of systemic ozone damage do seem to mimic the effects of ionizing radiation and since ozone apparently does damage lymphocyte DNA, one would predict that exposure to ozone should set off a round of repair synthesis in the circulating blood lymphocytes of the exposed animal. If this prediction held true, it would provide a valuable new probe for measuring the extent and mechanism of ozone damage. In the first place, the magnitude of the repair synthesis should be proportional to the extent of damage (11).

Secondly, by examining the repair process closely, one should be able to gather some information as to the mechanism by which ozone damaged the DNA. In this project, the extent of DNA repair synthesis in cultured lumphocytes was measured following exposure of rabbits, guinea pigs, and hamsters to ozone.

Of course, since ozone damage should be radiomimetic, one would expect the damaged DNA to contain an increased number of single strand breaks. Thus, it should also be possible to measure the effects of ozone on the DNA directly by looking for single strand breaks in the DNA. In this project the number of single strand breaks in the DNA of peritoneal exudate cells was measured by alkaline elution profile or by alkaline sucrose gradient following exposure of mice to ozone.

SECTION 2

CONCLUSIONS

This assay technique does appear to be sensitive enough to detect DNA damage caused by the exposure of the whole animal to ozone. Clearly exposure of the mice to 5 ppm (9800 $\mu g/m^3$) ozone for 24 hours causes a significant increase in single strand breaks in the DNA of the leukocytes which are formed and which circulate through the peripheral blood during the ozone exposure. While this experimental result establishes the validity of the experimental approach, it does not provide any information on the likelihood of DNA damage occurring upon exposure to levels of ozone which are likely to be found in the environment. In the first place, exposure to 5 ppm ozone for a 24 hour period is extremely unlikely. Secondly, previous experiments have shown that normal defense mechanisms begin to be overwhelmed at 5 ppm.

While exposure to 1 ppm ozone for a 24 hour period is unlikely, exposure to levels approaching 1 ppm for shorter times does occur. In these experiments a 24 hour exposure to 1 ppm did appear to cause a slight decrease in the weight average molecular weight of the DNA in each of the four separate experimental trials. This suggests that some DNA damage in the form of single strand breaks was occurring. However, the difference in size distribution was rarely statistically significant. Thus, these experiments suggest that some DNA damage may occur at lower levels of ozone exposure, but the technique is not sensitive enough to accurately determine whether damage occurs at environmentally significant doses.

Finally, the effect of 48 hours exposure to 1 ppm (1960 $\mu g/m^3$) was measured in a single experiment. In this case, no decrease in weight average molecular weight of the DNA was noted following ozone exposure. The reasons for this could be twofold: 1) Most of the leukocyte accumulation in the peritoneal cavity will have occurred by 24 hours. By 48 hours, a repair synthesis may have ligated the damaged DNA. 2) By 48 hours the cell population which accumulates in the peritoneal cavity is more heterogeneous. The other cell types which accumulate may be less sensitive to ozone-caused DNA damage.

SECTION 3

RECOMMENDATIONS

This study still leaves open the possibility that ozone may damage the DNA of peripheral blood cells. As more sensitive techniques for assaying DNA damage become available, this question should be reinvestigated.

SECTION 4

EXPERIMENTAL PROCEDURES

The experimental protocol is summarized in Figure 1. Six adult male CD-1 mice (Charles River Breeding Labs, Wilmington, Mass.) (approximately 25 gm each) were color coded and weighed. The mcie were kept in two separate cages and fed water and standard rations ad libitum throughout the experiment. At time zero each mouse was injected with 1 ml of 0.2% glycogen into the peritoneal cavity. At +27, +30, +33, and +36 hours each mouse was injected with 2 mCi of ^{3}H-thymidine (40-60 Ci/mmole, obtained from New England Nuclear) per gm body weight via tail vein. One cage of mice was placed in an ozone chamber and exposed to either 1 ppm or 5 ppm ozone from +27 to +51 hours. Exposure to ozone was carried out in the inhalation toxicology facility at the Environmental Protection Agency, Research Triangle Park, NC. Ozone concentrations were monitored by chemiluminescence techniques (10). Exposure chambers and ozone generating system have been described previously (11,12). The other cage of mice (sham treated) was placed in an identical chamber and exposed to clean filtered ambient air. At +51 hours the mice were removed from the chambers and sacrificed by cranial-spinal separation. The peritoneal cavity was quickly exposed. 5-7 mls of PBS (Phosphate Buffered Saline) was injected into the cavity and the abdomen agitated to prepare a cell suspension. A small hole was made in the cavity and as much of the cell suspension as possible removed with a pasteur pipette.

The cells were then washed twice in cold PBS and pelleted at 500 x G for 10 min. in a clinical centrifuge at 4°C. The cells were then resuspended in 0.024 M EDTA, 0.075 M NaCl (pH 7.4) to a concentration of 5×10^{6}/ml. To 200 µl of such a cell suspension (1×10^{6} cells), 200 µl of 1.0% Triton, 200 mM NaCl, and 20 mM EDTA was added to give a final concentration of 0.5% Triton, 100 mm NaCl, and 10 mm EDTA. This suspension was incubated for 2 min at 37°C to lyse the cells. Next 200 µl of 0.9 M NaOH and 3 M NaCl was added to the lysate and mixed in by very gently turning the test tube. This mixture was incubated for 30 min at 37°C. One alkaline lysate was prepared for each mouse.

The alkaline lysate was then very gently poured onto the top of a 30 ml 5% to 20% sucrose gradient containing 0.9 M NaCl, 0.3 M NaOH, and 0.33% Triton. 200 µl of a standard DNA solution ($1.5A_{260}$ units of λ DNA and $1.5A_{260}$ units of T4 DNA - obtained from Miles Biochemicals) in .9 M NaCl, 0.3 M NaOH, and 0.33% Triton was also gently layered onto the top of the gradients. The gradients were then balanced by layering mineral oil on the top and centrifuged for 3 hrs at 25,000 rpm at 25°C in an SW27 rotor. The gradients were fractionated into 1.5 ml fractions. After at least 1 hr at 4°, the samples were filtered through glass fiber filters (Whatman 934AH). The filters were rinsed successively with cold 5% TCA, 0.1 M HCl, and 95% ethanol. They were then dried and counted in toluene scintillation fluid.

The molecular weight of each fraction of DNA was determined from a semilog plot of molecular weight versus fraction number based on the position of the DNA standards in that run (λ_{DNA} = 32 x 10 daltons and T4 DNA = 120 x 10 daltons). The weight average molecular weight ($\bar{M}_w$) of the DNA in each gradient was determined from the equation:

$$\bar{M}_w = \frac{f_1M_1 + f_2M_2 + \ldots f_iM_i}{100}$$

where f_1, $f_2 \ldots f_i$ = percent of total DNA in that gradient fraction

M_1, $M_2 \ldots M_i$ = molecular weight of that gradient fraction

The number average molecular weight ($\bar{M}_n$) was assumed to be 0.5 times the weight average molecular weight as shown by Ono and Okada (19). The number of single strand breaks caused by the ozone exposure was calculated as described by Ono and Okada (19). That is:

$$\begin{array}{l}\text{\# of single strand} \\ \text{scissions per } 10^{12} \\ \text{daltons of DNA}\end{array} = 10^{12} \times \frac{1}{\bar{M}_n\ (O_3\ \text{DNA})} - \frac{1}{\bar{M}_n\ (\text{sham DNA})}$$

SECTION 5

RESULTS

The primary goal of these experiments was to determine whether exposure to environmentally realistic ozone levels could damage the DNA of cells peripheral to the lung tissue. Much of the effort, however, went into the development of a suitable animal model system. Obviously, for such a model system to be useful, whole animal exposure to ozone was desirable. Previous studies had suggested that red blood cells and lymphocytes of peripheral blood were affected by ozone exposure (see Introduction). Because of this and the ease of obtaining and analyzing peripheral blood samples, peripheral blood cells were obtained shortly after ozone exposure and analyzed for damage done to the DNA. In the initial experiments, rabbits were used as the experimental animal and DNA repair synthesis was measured in peripheral blood lymphocytes using essentially the technique of Clarkson and Evans (14). The rationale for such an experimental approach was based on the previous observations that the magnitude of DNA repair synthesis in human lymphocytes was known to be proportional to the extent of DNA damage following cellular exposure to UV irradiation (13) and to a number of known mutagens (14,15). Unfortunately, the rabbit lymphocytes did not show measurable levels of DNA repair synthesis following exposure to either 1 ppm ozone for 24 hours or UV irradiation. Guinea pig and hamster lymphocytes were similarly negative for DNA repair synthesis. Rats and mice did not provide sufficient lymphocytes for the DNA repair synthesis assay. Subsequent discussions with other workers in the field confirmed that they also had found DNA repair levels in most rodent lymphocytes were too low to successfully carry out this type of experiment, although that information is not discussed in the literature.

The next approach was to measure DNA damage directly, using sedimentation on alkaline sucrose gradients as a measure of DNA size (Zuboff and Sarma (16)). If the ozone caused single strand DNA breaks (which would be the expected response). There should be a measurable decrease in the size of the DNA on alkaline sucrose gradients. Rabbit lymphocytes were used in this study. The main difficulty for this kind of experiment is that it is almost impossible to label the lymphocyte DNA sufficiently in situ to be able to analyze that DNA on an alkaline sucrose gradient. To circumvent this problem the DNA was measured spectrofluorometrically using the assay of Kissane and Robins (17). Unfortunately, this method did not probe to be sensitive enough to measure the lymphocyte DNA without overloading the gradient. The principle reagent (3,5-diaminobenzoic acid) also reacted with lipid components of the cellular lysate, thus reducing the reproducibility of the assay. Although several attempts were made to improve the assay, this approach eventually had to be abandoned.

Next the elution profile technique of Kohn et al (18) was used to measure single strand breaks. This technique yielded enough DNA to be measured accurately with the fluorometric assay. Unfortunately, the elution profile was too broad to allow accurate measurement of single stranded DNA breaks. No significant difference was observed even after exposing isolated lymphocytes to 1 ppm ozone for 3 hours.

The final model system used involved analysis of peritoneal exudate cells (PECs) consisting mostly of polymorphonuclear leukocytes (PMNs). This system was chosen because following injection of a glycogen solution into the peritoneal cavity sufficient PMNs and other leukocytes are produced in the bone marrow and released into the bloodstream to allow in situ labeling of the DNA. These PECs circulate through the bloodstream for a brief period of time during which they are exposed to ozone and then accumulate in the peritoneal cavity where they can be readily isolated. The labeled DNA from these PECs can then be analyzed on an alkaline sucrose gradient for single strand breaks. The general protocol for such an experiment is shown in Figure 1. The injections of ^{3}H-thymidine must be given repeatedly at well defined intervals following the glycogen injection to provide for optimal labeling of the DNA. An extended ozone exposure time was chosen to allow maximum chance of exposing circulating PECs to ozone.

The first four experiments were carried out at 5 ppm ozone for 24 hours. It was felt that this level of ozone exposure should provide maximal DNA damage and thus serve to indicate whether or not this experimental protocol was sensitive enough to detect ozone related damage. A typical set of alkaline sucrose gradients of DNA obtained from ozone and sham exposed animals is shown in Figure 2. The ozone treated DNAs are located closer to the top of the gradient, indicating slower sedimentation and smaller molecular weight. The means and standard errors of the molecular weights are shown for each experiment in Table I. Bartlett's test (20) of equal variances was rejected so that analysis of variance could not validly be used to analyze the raw data. Because the variances did not appear to vary in any systematic fashion and because the heterogeneity was probably partially attributable to small sample sizes. Nonparametric tests were employed. Results from the Wilcoxon two-sample test indicated no statistically significant difference between the sham and ozone means at the .01 level. Thus, exposure to 5 ppm ozone for 24 hours resulted in detectable DNA damage.

Next, four experiments were carried out at an exposure level of 1 ppm ozone for 24 hours. A typical set of alkaline sucrose gradients is shown in Figure 3. The means and standard errors for the molecular weights are shown in Table I. Once again, using Bartlett's test. The hypothesis of homogeneous variances was rejected. Results from the Wilcoxon two-sample test indicated no statistically significant difference between the sham and ozone means.

Finally, one experiment was carried out at an exposure of 1 ppm ozone for 48 hours. The alkaline sucrose gradients are shown in Figure 4. The molecular weight means and standard errors appear in Table III. The Wilcoxon test indicated no statistical difference in molecular weights between the control animals and those exposed to ozone.

FIGURE LEGENDS

Figure 1: Outline of the Experimental Protocol

Figure 2: Alkaline Sucrose Gradient Profiles of DNA from Three Ozone and Two Sham Exposed Animals (5 ppm for 24 hours)

Mice were exposed to either ambient atmosphere or 5 ppm ozone for 24 hours as outlined in Figure 1. Peritoneal exudate cells were collected from each animal and their DNA analyzed on alkaline sucrose gradients as described in Experimental Procedures. In this experiment, sufficient DNA for analysis was obtained from only two sham exposed animals. The top and bottom of the gradient along with the sedimentation positions of the DNA standards (λDNA = 32×10^6 daltons and T4 DNA = 120×10^6 daltons) are indicated for each set of gradients. The solid, dotted, and dashed lines indicate duplicate gradients run with either the ozone treated or sham DNA.

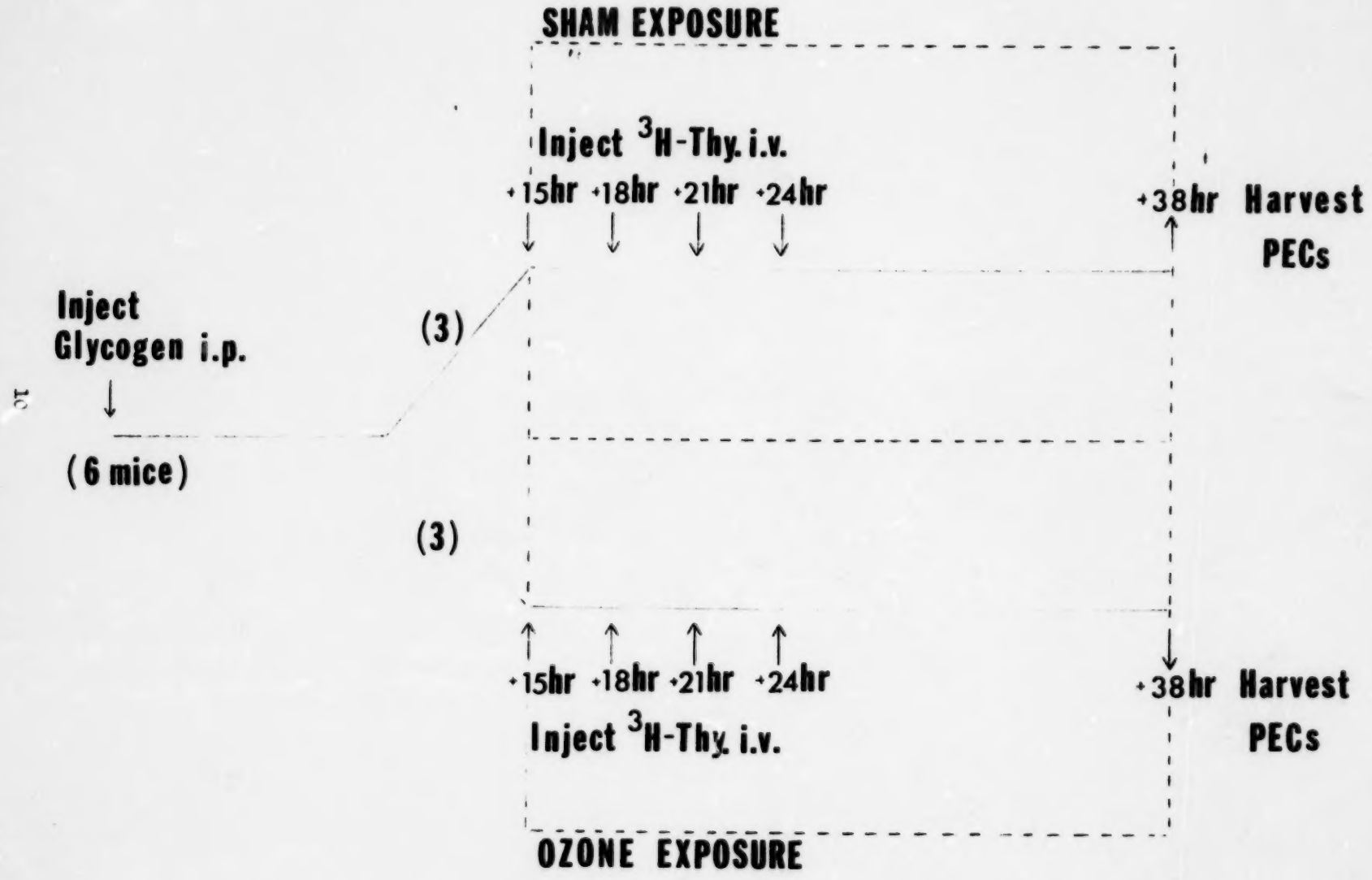
Figure 3: Alkaline Sucrose Gradient Profiles of DNA from Three Ozone and Two Sham Exposed Animals (1 ppm for 24 hours)

The experimental conditions were the same as in Figure 2 except that the exposure was to 1 ppm ozone for 24 hours.

Figure 4: Alkaline Sucrose Gradient Profiles of DNA from Three Ozone and Two Sham Exposed Animals (1 ppm for 48 hours)

The experimental conditions were the same as in Figure 3 except that the exposure was to 1 ppm ozone for 48 hours. The injection of ^{3}H-thymidine (2 mc/mg) were given at +15 hrs, +18 hrs, +21 hrs, +24 hrs, +38 hrs, +42 hrs, +46 hrs. The ozone exposure was from +15 hrs to +63 hrs. All other conditions were the same except that the gradients were fractionated into 1 ml aliquots rather than 1.5 ml aliquots.

FIGURE 1


SHAM EXPOSURE
Inject ^{3}H-Thy. i.v.
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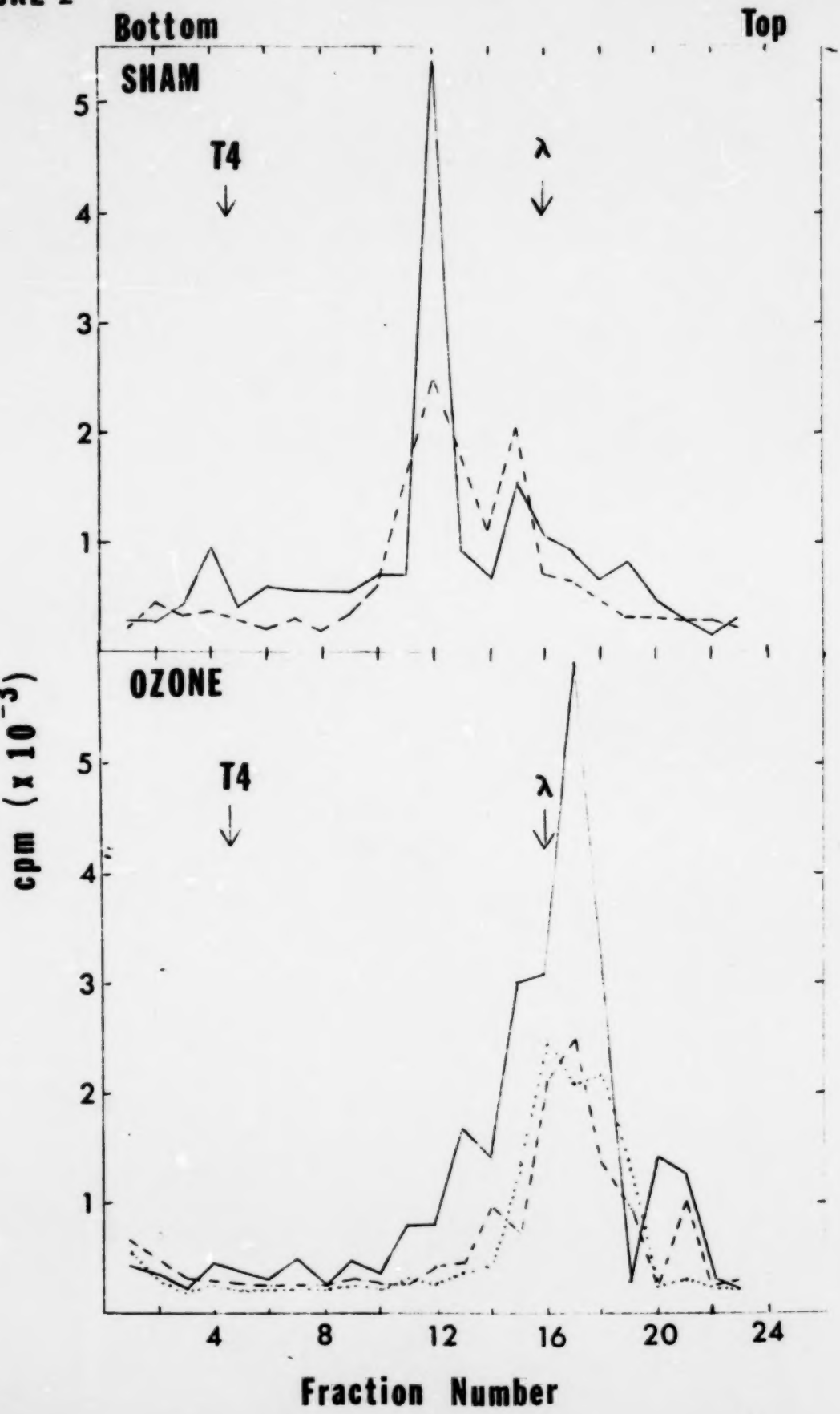
+38hr Harvest PECs
Inject Glycogen i.p.
(6 mice)
(3)
(3)
+15hr +18hr +21hr +24hr
Inject ^{3}H-Thy. i.v.
+38hr Harvest PECs
OZONE EXPOSURE

FIGURE 2


Bottom
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Fraction Number

FIGURE 3

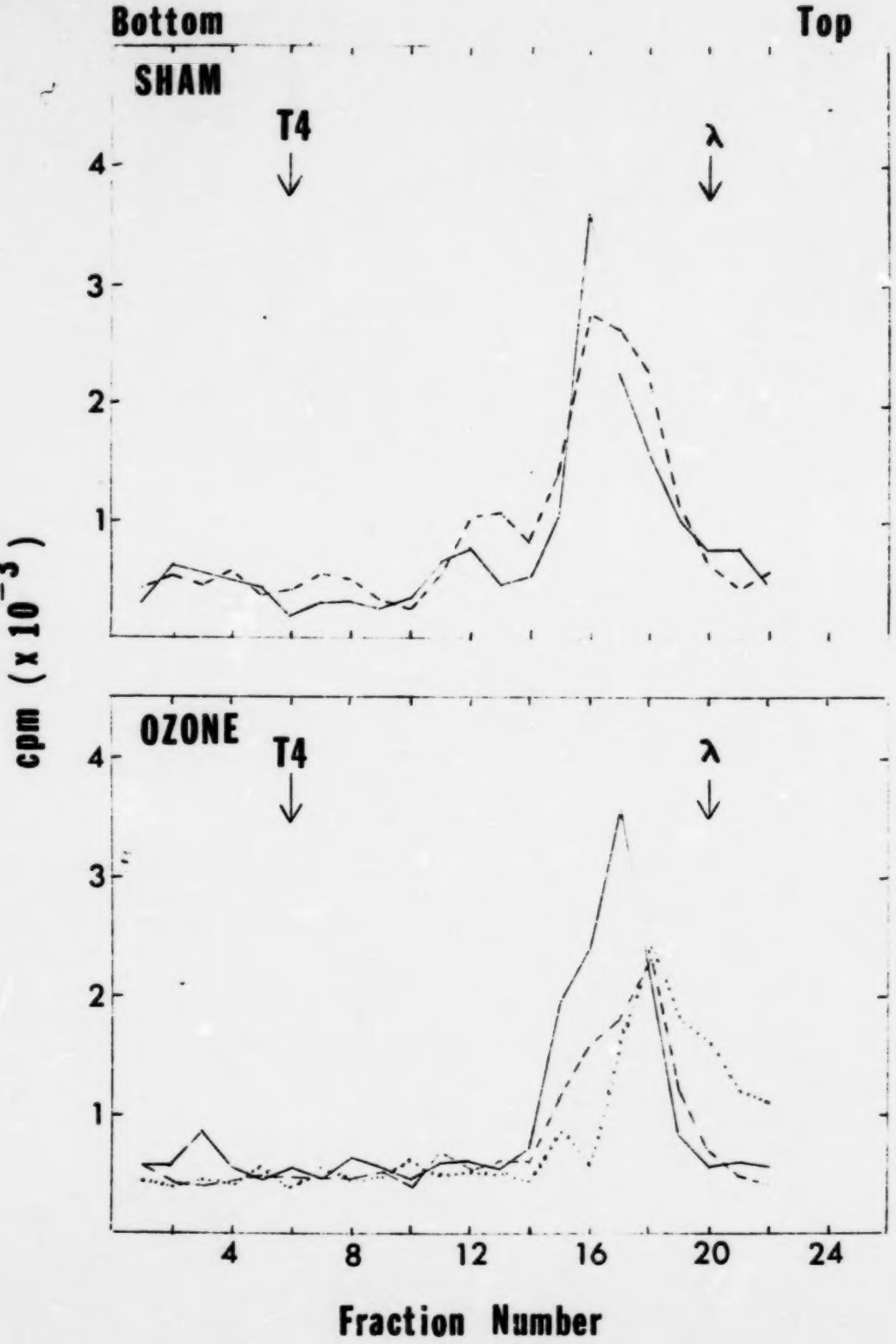
Bottom
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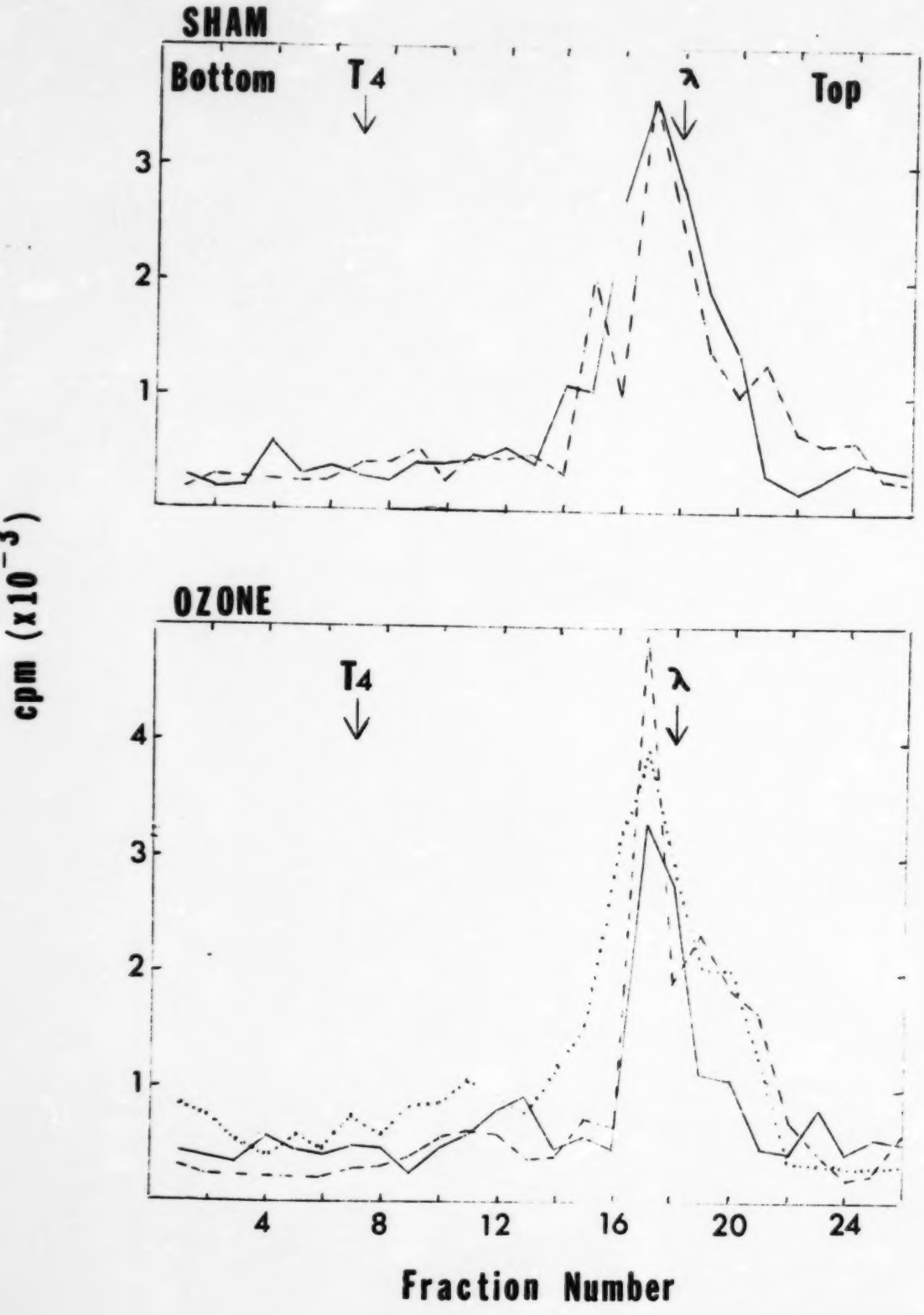
FIGURE 4


SHAM
Bottom
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Top
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OZONE
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8
12
16
20
24
cpm (x10⁻³)
Fraction Number

Table I. Weight Average Molecular Weights and Number of Single Strand Breaks Following Exposure to 5ppm Ozone for 24 hours

Exp #	$\bar{M}_W$ sham ($\times 10^6$) $\bar{X} \pm$ s.e. (N)	$\bar{M}$ ozone ($\times 10^6$) $\bar{X} \pm$ s.e. (N)	# of single strand breaks caused by ozone per 10^{12} daltons of DNA ($\times 10^5$) $\bar{X} \pm$s.e. (N)
1.	5.30±.17 (3)	4.58±.03 (3)	.6±.03 (3)
2.	5.03±.16 (3)	3.95±.21 (3)	1.1±.3 (3)
3.	4.85±.15 (3)	3.40±.06 (2)	1.8±.1 (2)
4.	6.27±.32 (2)	4.85±.26 (3)	1.0±.2 (3)

The weight average molecular weights ($\bar{M}_W$) and number of single strand breaks were determined from the sucrose gradient profile as described in Experimental Procedures. Wilcoxon two-sample test results determined the sham and ozone means ($\bar{M}_W$) to be significantly different at the .0001 level.

Table II. Weight Average Molecular Weights Following Exposure to 1 ppm Ozone for 24 Hours

Exp #	$\bar{M}_w$ (sham) ($\times 10^6$)	(n)	$\bar{M}_w$ (ozone) ($\times 10^6$)	(n)	Standard Error of the Difference	Standard Normal Variable	Probability that the two $\bar{M}_w$ are actually identical
1	5.16 ± 1.0	(3)	5.01 ± 1.1	(3)	0.87	0.172	0.43
2	4.95 ± .01	(2)	4.82 ± .97	(3)	0.56	0.23	0.41
3	3.94 ± .25	(2)	3.51 ± .01	(3)	0.18	2.43	0.007
4	5.72 ± .06	(3)	5.68 ± .54	(3)	0.32	0.126	0.45

The weight average molecular weights were determined from the sucrose gradient profiles as described in Experimental Procedures. The numbers above for $\bar{M}_w$ are usually the mean and standard deviation of the size of the DNA from three separate animals run on three gradients. The actual number of animals analyzed is indicated in parentheses. The sucrose gradient profiles for Experiment 2 are shown in Figure 3.

Table III. Weight Average Molecular Weights Following Exposure to 1 ppm Ozone for 48 Hours

Exp #	$\bar{M}_w$ (sham) (x 10^6)	(n)	$\bar{M}_w$ (ozone) (x 10^6)	(n)	Standard Error of the Difference	Standard Normal Variable	Probability that the two $\bar{M}_w$ are actually identical
1	3.84 $\pm$ .11	(2)	3.92 $\pm$ .19	(3)	0.14	0.25	0.40

The weight average molecular weights were determined from the sucrose gradient profiles in Figure 4 as described in Experimental Procedures. The numbers of the $\bar{M}_w$ listed above are the mean and standard deviation of the size of the DNA from three ozone treated animals and two sham treated animals.

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